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14. ABSTRACT

Erythropoietin is effective in correcting the anemia associated with cancer and chemotherapy. However, Epo receptors (EpoR) have been found on tumor cells and Epo may stimulate these cells. We discovered that prostate cancer cell lines and primary prostate tumors express EpoR. In this study, we propose to gain insight into a pattern of EpoR expression in primary human prostate tumors and adjacent normal tissue and to study the role of the EpoR and the effect of Epo administration on growth of prostate cancer cells transplanted into SCID mice. We have prepared prostate cancer cell lines containing antisense EpoR constructs to be used in loss-of-function studies in vivo. Because we had found that the tetracycline inducible vector system was very "leaky", we transfected LNCaP and PC-3 cells with stable antisense constructs. We found that the single stranded cDNA probe was not sensitive enough and, therefore, tested an S1 nuclease protection technique. We completed our immunohistochemistry pilot study showing EpoR in primary human prostate tumors. We also determined of baseline growth kinetics for prostate cancer cells transplanted into SCID mice.

15. SUBJECT TERMS
cell biology, erythropoietin, receptor, proliferation, signal transduction, in situ hybridization,Immunochemistry, SCID mice

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INTRODUCTION

The factors regulating neoplastic transformation of the prostatic epithelium and those regulating prostate cancer growth and progression are poorly understood. Prostate cells, both normal and malignant, are known to respond to several hormonal and cytokine stimuli. We have now discovered that transformed and malignant human prostate cells express functional EpoRs and that Epo stimulates cell growth. Primary human prostate cancer specimens also express the EpoR gene. We hypothesize that endogenous Epo may serve as a previously unrecognized regulator of prostate cell biology. Moreover, the use of pharmacologic doses of rEpo in clinical practice, including the treatment of the anemia associated with cancer, may further influence disease progression. We expect that our results will provide important information about the role of the Epo/EpoR axis in normal and malignant prostate cell biology and pathobiology. Additionally, our results will lead to better decision-making regarding the use of rEpo in treating anemic cancer patients.

BODY

Experimental Procedures- New Techniques Developed

Western blots and phosphoSTAT5 analyses. Lysates from 1 x 10^5 cells - from BaF3, BaF3/EpoR, and from each of the five prostate cell lines (prepared in SDS gel sample buffer) - were heated at 95 ^oC for 2 min before being separated on a 10% SDS-polyacrylamide gel. Proteins from the gel were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes at 4^o C (75V, 4 hr). Membranes were blocked for 1 hr with 5% nonfat milk powder in PBS - 0.1% Tween 20 for 30 min at room temperature and incubated overnight at 4^o C with primary antibody to EpoR [1] diluted in PBS – 0.1% Tween20 - 1% nonfat dry milk. Alkaline phosphatase-conjugated anti-rabbit second antibody (NEB 7051-1) was incubated with the blot for 2 hr at room temperature. Blots were developed with CDP-AP Western Detection Reagent (NEB) and exposed to Kodak X-ray film. Cell lysates/immunoprecipitates from signal transduction assay experiments were similarly electrophoresed and transferred to PVDF membranes. Washed and blocked membranes were incubated overnight at 4^o C with primary antibodies that included phospho-STAT5 (Advantex AX-1) and STAT5 (Santa Cruz C-17), as detailed for the individual experiments, followed by incubation with second antibody and detection as above. Gel-Pro Analyzer^{TM} software (version 3.1, Media Cybernetics) was used to quantify the phosphoSTAT5b bands.

Immunohistochemistry Immunohistochemical characterization of fixed, embedded and sectioned cells or prostate tissue samples was performed as described [2], using EXO1 anti-EpoR primary antibodies from our laboratory [1]. All samples were counterstained with hematoxylin.

RT-PCR Cells were cultured to 80-90% confluence in 25 cm^2 flasks, medium was aspirated, and 3 ml of TRIzol reagent was added to each 25 cm^2 flask. Cell lysates in TRIzol reagent were stored at -80°C before RNA purification. RNA was separated by adding 0.6 ml of chloroform, mixing, and centrifugation at 12,000 x g for 10 min at 4°C. RNA present in the aqueous phase was precipitated with 1.5 ml isopropanol, followed by centrifugation at 12,000 x g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol, dried at room temperature for 10 min. and dissolved in 50 µl diethyl pyrocarbonate (DEPC)-treated nuclease-free water. Resuspended RNA was quantified by spectrophotometric absorbance at 260 nm.

EpoR PCR primers were selected as described [3]. The sense primer selected for human EpoR is (5'-ACCGTGTCATCCACATCAAT-3'), corresponding to nucleotide number 537 to 556 of NCBI sequence NM_000121; and the antisense primer selected for EpoR is (5'-GCCTTCAAACTCGCTCTCTG-3'),

corresponding to nucleotide number 1002 to 1021. The calculated PCR product DNA is 485 bp in length. We also selected primer pair (5'-AAGGCTGAGAACGGGAAGCTT-3'), corresponding to nucleotide number 241 to 261, and (5'-TCCACCACCCTGTTGCTGTA –3'), corresponding to nucleotide number 1018 to 1037, to co-amplify a 797 bp fragment from the GAPDH gene, as control.

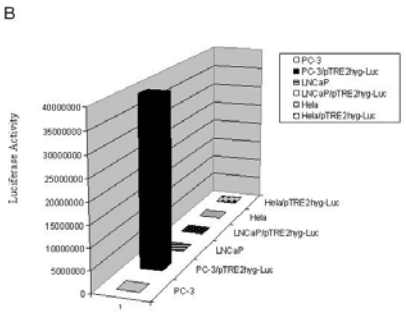
RT-PCR was performed using a one-step RT-PCR kit (QIAGEN) and a programmable thermal cycler (GeneAmp System 2400, Perkin Elmer Life Sciences). Each reaction mix contained 5µl of QIAGEN one-step RT-PCR 5X buffer, 1µl of deoxynucleoside triphosphates (dNTP, 10mM each), 1.5µl of 10µM EpoR forward primer, 1.5µl of 10µM EpoR reverse primer, 1µl of 2µM GAPDH forward primer, 1µl of 2µM GAPDH reverse primer, 1µl of template RNA (200ng), 1µl of Qiagen RT-PCR enzyme mix, and 12µl nuclease free water. The mixture was incubated at 50°C for 30 min., 95°C for 15 min., and followed by 35 cycles at 95°C for 1 min., 56°C for 1 min, and 72 °C for 35 second. The final elongation step was extended for an additional 8 min. The resulting PCR fragments (10 µl of the total reaction) were resolved on 1.2% agarose gel in TBE buffer (89 mM TrisHCl – 89 mM boric acid – 2 mM EDTA, pH 8.3) and visualized with ethidium bromide staining.

Progress Made on Each Task

Task 1: Establish cell lines for loss-of-function experiments.

We began by subcloning EpoR cDNA into the stable expression vector PCDNA3.1 and, separately, into the inducible expression vector Tet-On. We confirmed the proper orientation and in frame insertion into the vectors by DNA sequence analysis. Importantly, we discovered that the Tet-On system is quite leaky, that is, it exhibits substantial expression of the inserted transgene even in the absence of tetracycline (Figure 1).

We carried out a series of experiments on the Tet-On vector itself and discovered that the kanamycin resistance cassette contained a promoter sequence that activates the downstream gene, resulting in constitutive expression. We determined that deletion of this kanamycin cassette results in a highly regulatable silent vector in the absence of tetracycline induction (Figures 2-4).



B

Luciferase Activity of Cells Transfected with or without pTRE2hyg-Luc plasmid

	PC3		LNCaP		Hela	
	Mock	pTRE2hygluc	Mock	pTRE2hygluc	Mock	pTRE2hygluc
Well 1	258	47502770	323	10845	234	807
Well 2	325	34907016	256	14957	342	712
Well 2	276	36579806	245	12368	233	756
Average	286	39663197	275	12723	270	758

Figure 1. Leaky Tet-On system. Note high luciferase activity exhibited by Tet vector in the absence of inducer.

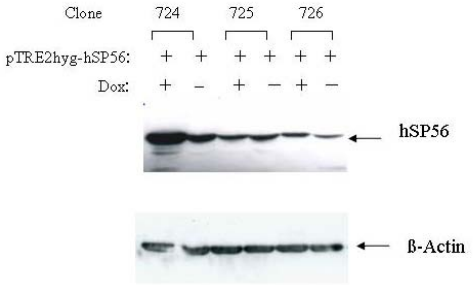


Figure 2. High hSP56 expression in the absence of presence of doxycycline inducer.

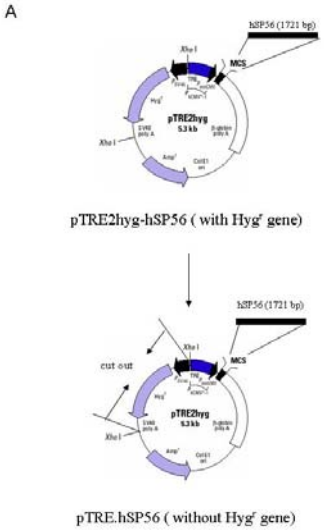


Figure 3. Structure of original Tet-On vector (top) and engineered vector with *Hyg* removed (bottom).

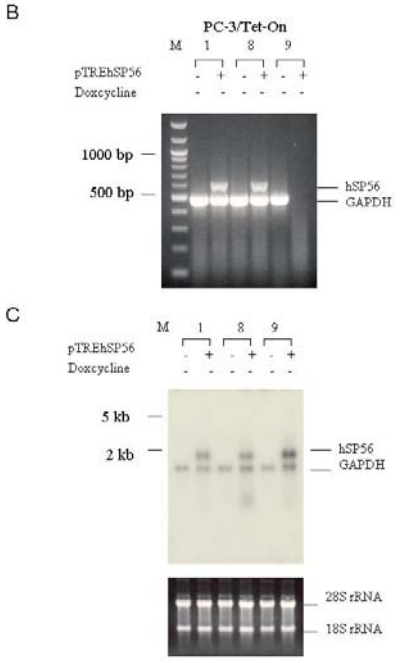


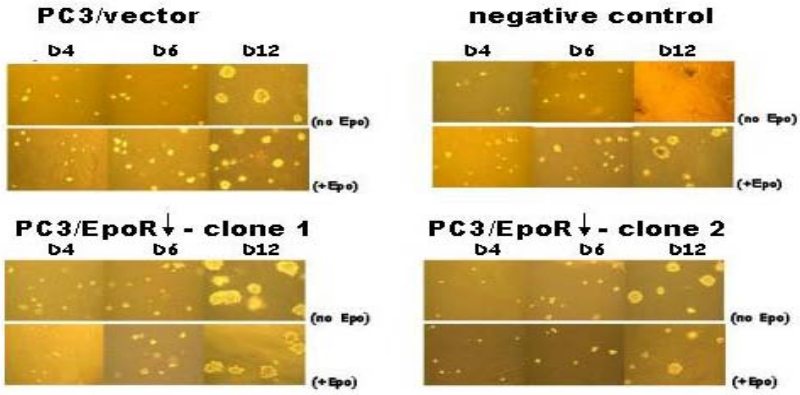
Figure 4. Vector with *Hyg* removed is now highly inducible by doxycycline.

Next we transfected both LNCaP and PC-3 cell lines with the stable antisense constructs. Antibiotic selection resulted in several resistant clones consistent with expression of the insert. Because the vector encodes an antisense strand of EpoR message, both conventional northern blot using cDNA probe and real time PCR failed to discriminate between expression of the sense EpoR messenger mRNA from the antisense mRNA.

Therefore, we designed a single stranded cDNA probe that is specific for the endogenous EpoR mRNA and are preparing to carry out the appropriate northern blot analyses. This approach is significantly less sensitive

than real time PCR or even conventional northern using cDNA. We were not able to detect EpoR mRNA using this method and, therefore, were not able to confirm down-regulation of EpoR by the antisense construct. Next, we moved forward with a S1 nuclease protection assay. It, too, proved insufficiently sensitive. Finally, an RT-PCR method using a two-step approach with strand specific nested primers that appeared promising initially ultimately failed. We concluded that we would have to prepare new cell lines with EpoR down-regulated using RNAi methodology. The funding period and extension ended before we were able to pursue this.

We tried to downregulate EpoR in PC3 and LNCaP cells by stable transfection with the full-length EpoR cDNA "antisense construct" [4], to create cell lines PC3/EpoR↓ and LNCaP/EpoR↓, respectively. We have shown that EpoR is <u>functionally downregulated</u> in at least 2 of the PC3/EpoR↓ subclones. 10^5 cells for each PC3/EpoR↓ clone were plated in two-layer soft agar cultures, in the absence (top row, each set of photographs) or presence (bottom row) of 10 U Epo/ml. Colonies were photographed on days 4, 6, and 12. *Top left*: PC3 cells transfected with empty pcDNA3.1 vector ("mock" transfection): Colony size/number increased over time and colony number was greater in presence of Epo. *Top right*: one PC3/EpoR↓ clone in which transfection with the EpoR "antisense construct" did *not* result in successful downregulation of EpoR (i.e. colony size /number in culture increases in the presence of Epo, as in vector control). *Bottom, left and right*: two PC3/EpoR↓ clones in which addition of Epo to cultures does <u>not</u> increase colony size or number, suggesting that EpoR *was* successfully downregulated.



Task 2: To carry out a pilot study to determine the distribution of EpoR in primary tumors, metastatic foci and normal prostate tissue.

In the prior year, we used our polyclonal affinity purified rabbit anti-human EpoR antibody to optimize immunohistochemistry methods on the rodent BaF3 cell line stably transfected with the human Epo receptor and compare these results with non- transfected cells. As shown in Figure 5, cells lacking the EpoR have a relatively low background of antibody binding (brownish red pigment). In contrast, as shown in Figure 6, cells expressing the human EpoR are highly positive for antibody binding.

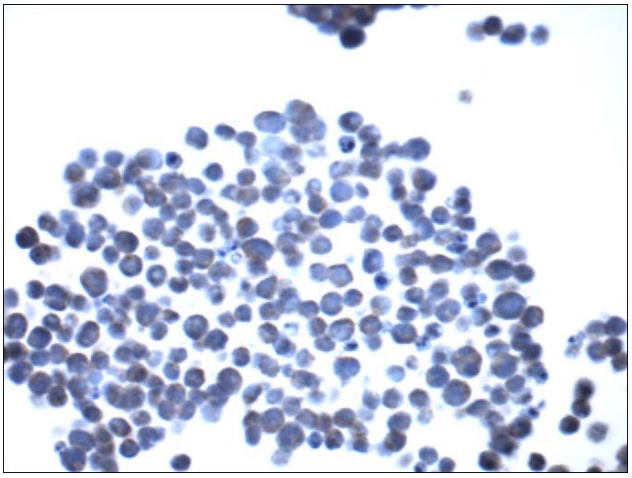


Figure 5. Control BaF3 cells stained with anti-EpoR antibody.

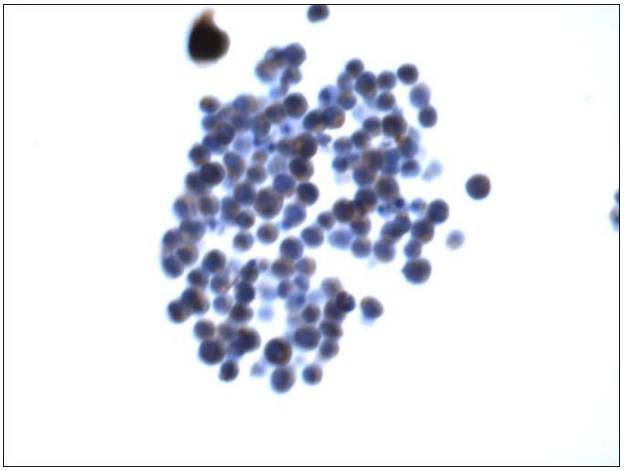


Figure 6. BaF3-EpoR cells stained positively with anti-EpoR antibody.

These conditions were then used to test for Epo receptor in human prostate cancer cell line PC-3. As shown in Figure 7, immunostaining in PC-3 was robust and heterogeneous.

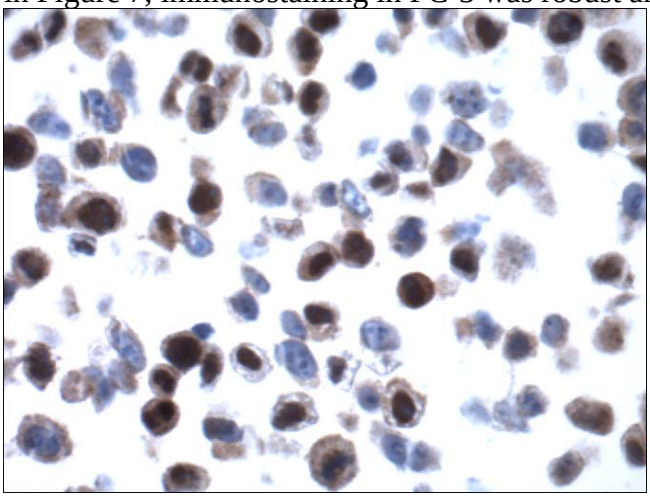


Figure 7. Robust EpoR immunostaining of PC-3 prostate cancer cells.

Finally, we carried out studies of fixed and embedded human prostate cancer primary tumors. We confirmed expression of Epo receptor by malignant prostatic epithelial cells in virtually all specimens examined. An example is shown in Figure 8. We found no discernable quantifiable difference in expression that correlated with Gleason grade. Therefore, no statistical analysis could be performed Similar results have been reported by other investigators [5]. Because of these results, we decided not to proceed with *in situ* hybridization, since it would offer no advantage over immunohistochemistry.

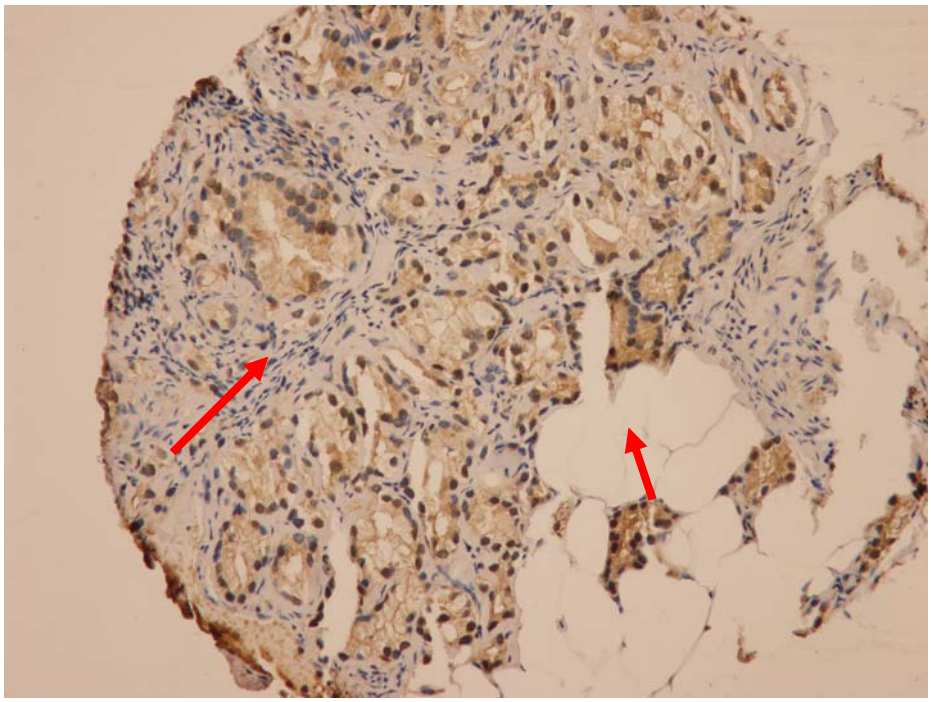


Figure 8. Positive EpoR immunostaining of malignant prostatic epithelium in a primary human prostate cancer. Red arrows point to two of many positive areas. Stroma is negative.

Task 3: To study the role of the EpoR and the effect of recombinant Epo administration on the growth and metastasis of human prostate cancer cells transplanted into SCID mice.

We completed the determination of baseline growth kinetics for nontransfected wildtype LNCaP and PC-3 cells transplanted subcutaneously into SCID mice (Figure 9).

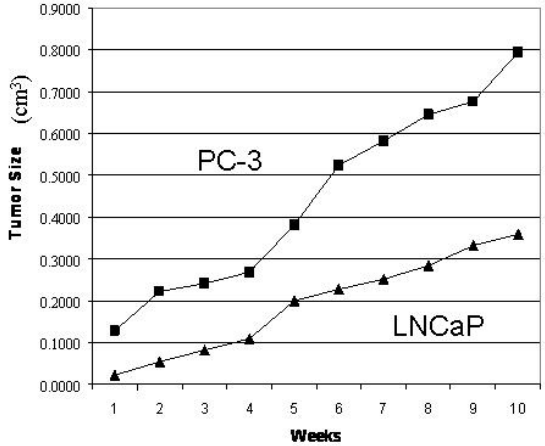


Figure 9. Growth kinetics for PC-3 and LNCaP cell tumors transplanted SQ in SCID mice. Points are the means of 8 mice in each group.

We also established the orthotopic transplantation methodology (Figure 10).

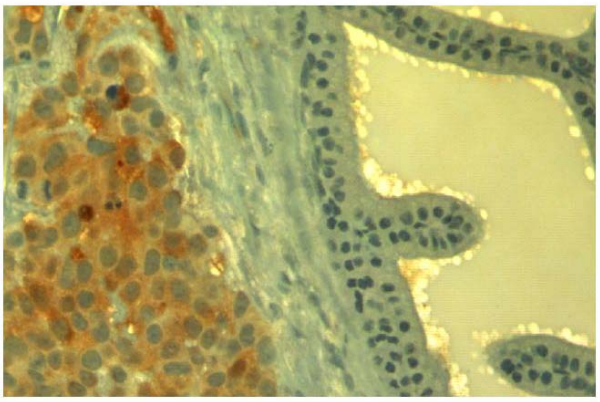


Figure 10. LNCaP tumor cells (left) invading SCID mouse prostate (right) after orthotopic transplantation. LNCaP are stained (reddish brown) for a cytoplasmic protein using an Ab that specifically recognizes the human but not the mouse homologue.

Because the funding period ended, we did not finish this third task.

Other Progress

Establishment of stable PC-3 cells with knocked-down EpoR expression

Because we could not confirm downregulation of EpoR transcript due to our use of a full-length antisense EpoR construct (see above), we elected to re-engineer the EpoR downregulated cell lines using RNAi technology. The RNAi Consortium (TRC) human shRNA clones targeting EpoR were purchased from Open Biosystems. Each bacterial clone contains a lentiviral vector (pLKO.1) designed to express a hairpin of 21 bp sense and antisense stem and a 6 bp loop targeting a coding region of EpoR mRNA (accession number NM_000121). The clone IDs and target sequence information are listed in Table 1, together with control plasmids used in this study, pLKO (empty vector, Sigma) and pLKO-NTC (non-targeting control plasmid, Sigma).

Table 1. The shRNA information for EpoR down-regulation.

Clone	TRC ID	Target Sequence (5'-3')	Target Region* (in bp)
pLKO-EpoR1	TRCN0000058313	CGTGTCATCCACATCAATGAA	539-559
pLKO-EpoR2	TRCN0000058314	CCCTTATGAGAACAGCCTTAT	1597-1617
pLKO-EpoR3	TRCN0000058315	CACCTAAAGTACCTGTACCTT	1487-1507
pLKO-EpoR4	TRCN0000058316	TGCCAGCTTTGAGTACACTAT	1399-1419
pLKO	N/A	No insert	N/A
pLKO-NTC	N/A	CAACAAGATGAAGAGCACCAA	Non-targeting

* The target regions of pLKO-EpoR are the nucleotide numbers corresponding to EpoR mRNA sequence (NM_000121). All four pLKO-EpoR constructs target the coding region (137-1663 bp) of EpoR mRNA. N/A; not-applicable.

PC-3 cells were transfected with the shRNA constructs using FuGENE6 transfection reagent. The cells were resuspended in the culture media containing 4 ug/ml of puromycin 48 hrs after transfection, and plated in a 96-well dish. The puromycin-resistant single colonies were propagated to prepare the frozen stocks and to determine the EpoR expression levels using semi-quantitative reverse-transcription PCR (sqRT-PCR).

sqRT-PCR for determining the EpoR expression levels

Total RNA was isolated from the individual clones at their sub-confluent states using RNAqueous-4PCR (Ambion). Purified RNA was treated with DNase I to remove genomic DNA contamination, reverse transcribed using RETROscript (Ambion) with random decamer as primer. Amount of cDNA corresponding to 10 ng of RNA was each used as template for semi-quantitative PCR (sqPCR) analysis.

The EpoR expression levels were determined by the PCR using the sense primer (5'-gcgatatcaccgtgtcatccacat, 529-552 bp of EpoR mRNA) and the antisense primer (5'-attctggtacagccacagctggaa, 1046-1069 bp of EpoR mRNA). The primers were designed to include intron

region, so that the PCR product from genomic DNA contamination, if any, can be distinguished by size difference. The levels of GAPDH in the same templates were also determined to be used as control by the PCR using the sense primer (5'-aaggctgagaacgggaagctt, 241-261 bp of GAPDH mRNA) and the antisense primer (5'-tccaccaccctgttgctgta, 1018-1037 bp of GAPDH mRNA).

A modified touch-down PCR cycle was used; for EpoR amplification, 94°C/3min, 20 cycles of 94°C/30sec-70°C (-1°C per cycle)/30sec-72°C/30sec, 10 cycles of 94°C/30sec-50°C/30sec-72°C/30sec, 72°C/5min; for GAPDH amplification, 94°C/3min, 20 cycles of 94°C/30sec-70°C (-1°C per cycle)/30sec-72°C/1min, 17 cycles of 94°C/30sec-50°C/30sec-72°C/1min, 72°C/5min. The amount of cDNA and the number of cycles used for the PCR were previously determined for the band intensity of amplified DNA to be in a linear range of detection. The effective number of cycles was twenty for EpoR and twenty-five for GAPDH, based on the melting temperature of the primers.

PC-3, PC-3/V (transfected with vector) or PC-3/NTC (transfected with non-targeting control plasmid) all showed EpoR products at the expected size (541 bp), while BaF3, not expressing human EpoR, did not (Figure 11). The similar levels of GAPDH product were amplified throughout the samples, indicating the amount of cDNA used in the sqPCR was similar. The strongest down-regulation of EpoR was observed in PC-3/EpoR^{KD}2C10, followed by 4B6. These stable cell lines were subjected to the further examination.

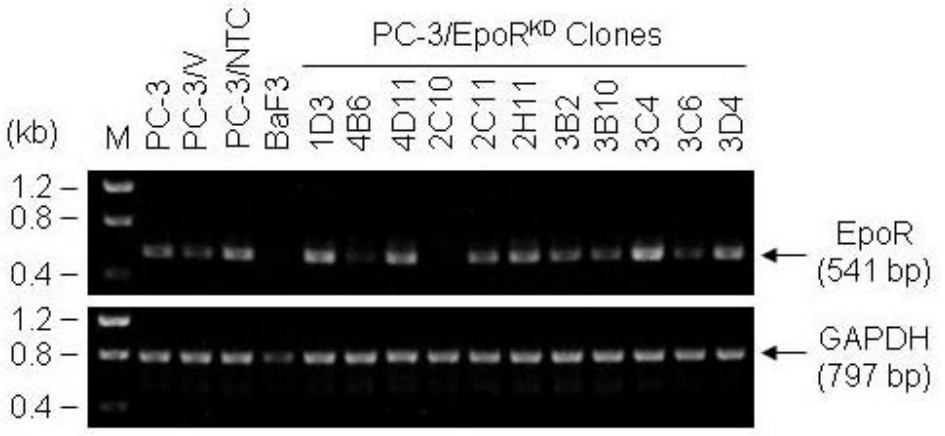


Figure 11. Semi-quantitative RT-PCR to determine EpoR expression levels in PC-3 cells stably transfected with EpoR shRNA constructs (PC-3/EpoRKD). Cells transfected with none (PC-3), with vector only (PC-3/V) and with non-targeting control plasmid (PC-3/NTC) were included as positive controls. BaF3, not expressing human EpoR, was used as negative control. DNA size marker (M) is labeled to the left and the expected sizes of EpoR and GAPDH products are indicated to the right.

Demonstration that the EpoR is Functional in Prostatic Epithelial Cells and Prostate Cancer Cell Lines.

Transformed human prostatic epithelial cells and prostate cancer cell lines express EpoR

We examined three transformed human prostate epithelial cell lines and two human prostate cancer cell lines for the expression of EpoR. The transformed prostate epithelial cell lines were selected as a series, representing increasing deviation from normal prostate epithelial biology. 267B1 cells are derived from normal human fetal prostatic epithelium and were immortalized by transformation with the SV40 T antigen [6]; X/267B1 are cells of the 267B1 lineage that have been further transformed by multiple cycles of X irradiation [7]; and Ki/267B1 cells are derived from 267B1 cells that have been transfected with an activated Ki-ras oncogene [8]. Ki/267B1 cells are tumorigenic when injected into nude mice. Both LNCaP [9] and PC-3 [10] cells are derived from metastatic human prostate cancer. LNCaP is an androgen-responsive cell line, and PC-3 is an androgen-nonresponsive cell line.

RT-PCR was used to identify EpoR mRNA from each of the prostate cell lines. As seen in Figure 12A, a specific 485 bp EpoR fragment was amplified from the RNA of the positive control BaF3/EpoR cells, which express the human EpoR (lane 1), but not from parental BaF3 cells, which do not express human EpoR (lane 2). Importantly, an identical 485 bp EpoR gene fragment was amplified from each of the prostate cancer and the transformed prostate epithelial cell lines (Figure 12A, lanes 3-7). Co-amplification of a 797 bp fragment of the GAPDH gene from each sample was included as a control, to demonstrate equal RNA loading of the samples.

All prostate cell lines also express EpoR at the protein level (Figure 12B). Cell lysates from BaF3/EpoR cells, bearing the transfected human EpoR, expressed a prominent EpoR protein band (lane 1), while no EpoR protein was detected in lysates of the parental (nontransfected) BaF3 cells (lane 2). Importantly, each of the prostate cell lines (lanes 3-7) clearly expressed EpoR protein. The size of the human EpoR protein was approximately 62 kDa, consistent with the size reported for the non-glycosylated form of the EpoR in BaF3/EpoR cells [11] and similar to that reported for LNCaP and PC-3 cells [12].

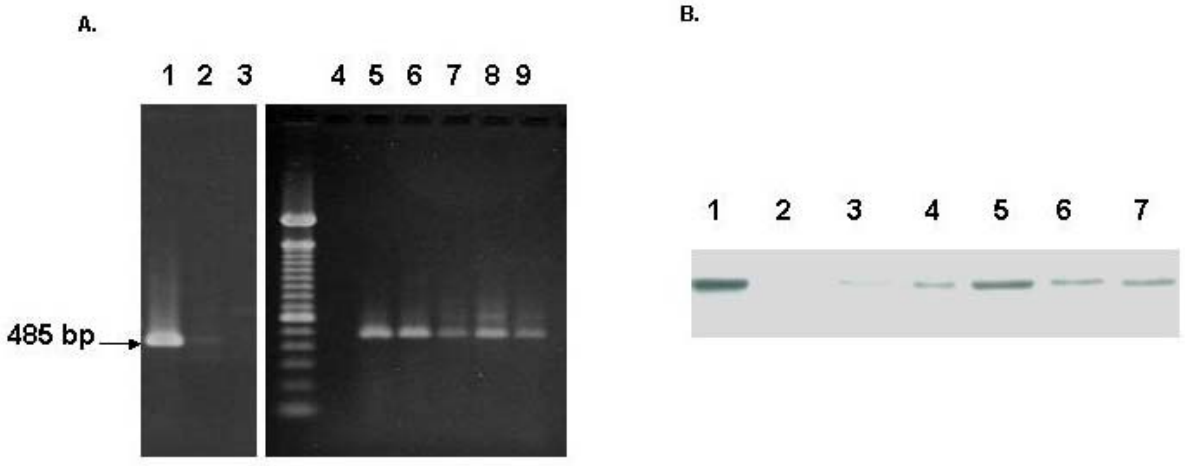


Figure 12. Human prostatic epithelial and prostate cancer cell lines express the EpoR gene and EpoR protein. (A) RNA from cells (as indicated) was used as template to co-amplify a specific 485 bp fragment of the human EpoR gene and a 797 bp fragment of the GAPDH gene using RT-PCR. Lane 1: BaF3 cells expressing the human EpoR (BaF3/EpoR); lane 2: BaF3 cells; lane 3: LNCaP; lane 4: PC-3; lane 5: 267B1; lane 6: X/267B1; lane 7: Ki267B1. (B) Lysates from 1 x 10^5 cells were separated electrophoretically on SDS-PAGE and western blots were developed with specific anti-EpoR antibodies. Lane 1: BaF3/EpoR (positive control); lane 2: BaF3 (negative control); lane 3: LNCaP; lane 4: PC-3; lane 5: 267B1; lane 6: Ki/267B1; lane 7: X/267B1.

Epo induces a dose-dependent growth response in human prostatic epithelial and prostate cancer cell lines

To determine whether these EpoR are functional, each of the five prostate cell lines was plated at low cell density in the absence or presence of specified concentrations of Epo, in RPMI 1640 containing 1% fetal bovine serum (FBS). After 72 hr, the Epo-dependent increase in cell growth was measured by quantifying the metabolism of MTT. As seen in Figure 13A, each cell line exhibited a dose-dependent proliferative response to Epo, at concentrations up to 100 U/ml. Figure 13B expands the low-dose portion of the curve and demonstrates that all of the lines exhibited at least modest-moderate growth stimulation at doses of Epo <10 U/ml and, in fact, 4 of the 5 lines showed growth stimulation by as little as 1 U Epo/ml. Figure 13C replots the data from Figure 2B to express the fold increase in cell growth in the presence of Epo and demonstrates clearly that cell growth was stimulated 1.5-3.5-fold by 1 U Epo/ml.

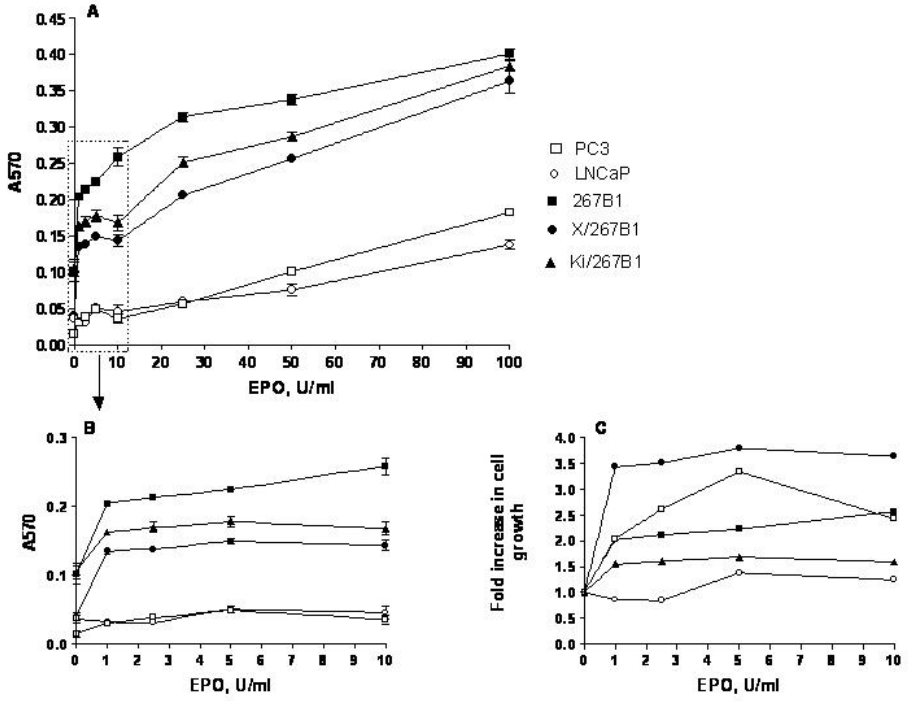


Figure 2

Figure 13. Prostate epithelial and prostate cancer cell lines proliferate in response to Epo. Panel A) Cells were plated under low-serum (1% heat inactivated FBS) conditions and incubated for 72 hr in the presence of increasing doses of rhEpo, from 0 to 100 U/ml. Cell proliferation was measured by absorbance at 570 nm using an MTT assay. Data are expressed as mean ± SEM of triplicate determinations and are presented with background (all reagents, minus cells) subtracted. In most cases the error bars fall within the symbol and, therefore, are not visible. Cell lines are: (□) PC-3, (O) LNCaP, (■) 267B1, (●) X/267B1, (▲) Ki/267B1. Panel B) The data from Panel A for the range of 0-10 U Epo/ml are expanded. Panel C) Data from Panel B are replotted to show the fold-increase in A570, as a measure of cell growth (growth in the absence of Epo set as 1.0 for each cell line).

Prostate cancer cells exhibit Epo-dependent signaling

The STAT5 proteins are established signal transduction intermediates for the Epo-dependent anti-apoptotic pathway in erythroid cells [13-19], and they appear to play an important role in prostate cancer cell survival and progression (Ahonen, 2003; Kazansky, 2003). Therefore, we examined STAT5 phosphorylation in prostate cancer cells in response to Epo stimulation. LNCaP and PC-3 cells were serum-starved and then treated with 50 U Epo/ml for 0-10 minutes. Although much lower Epo concentrations were effective in the

growth assay (Figure 13), this concentration was chosen because it is 5-10-fold higher than the saturable response concentration and should activate most or all of the EpoR simultaneously. Cell lysates were immunoprecipitated with anti-STAT5 antibodies, and western blots of the immunoprecipitated proteins were developed with anti-STAT5 or anti-phosphoSTAT5 antibodies. As seen in Figure 14, STAT5b was far more abundant than was STAT5a in both LNCaP and PC-3 cells (top panel). STAT5a was almost undetectable in PC-3 cells, consistent with published reports [20, 21]. Also, in both cell lines there was some baseline phosphorylation of the proteins, principally phosphoSTAT5a in LNCaP and phosphoSTAT5b in PC-3 (bottom panel). PhosphoSTAT5b levels in LNCaP cells (left, bottom) increased approximately 3.5-fold after 2.5 min of Epo stimulation and approximately 5.5-fold after 10 min Epo stimulation. In PC-3 cells (right, bottom), basal levels of phosphoSTAT5b were higher than in LNCaP, but the phosphoSTAT5b levels also were increased 1.8-fold and 2.0-fold after 2.5 and 10 minutes of Epo stimulation, respectively. Total STAT5b protein levels remained constant over this time (top panel).

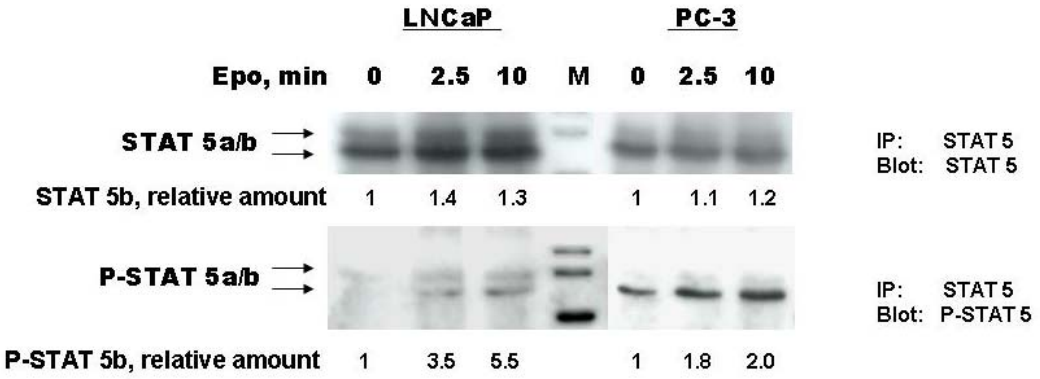


Figure 14. Epo triggers STAT5 phosphorylation in prostate cancer cell lines. Prostate cancer cell lines LNCaP (left) and PC-3 (right) were incubated in the absence or presence of Epo (50 U/ml) for the times indicated. Cell lysates were immunoprecipitated with antibodies to STAT5 protein and subjected to SDS-PAGE. Western blots were developed with STAT5 (top) or phosphoSTAT5 (bottom) –specific antibodies. M, molecular weight markers.

Normal and cancerous primary human prostate tissues express EpoR

In addition to human cell lines, we found that normal and cancerous primary human prostate tissue also express EpoR mRNA. RT-PCR was used to co-amplify a 485 bp EpoR fragment and a 797 bp GAPDH fragment from four paired cancerous primary human prostate tissue specimens. For each pair, N represents RNA derived from noncancerous areas and C represents RNA from cancerous regions of the same tissue sample. Figure 15 shows that each of the samples expresses the EpoR gene, albeit at varying levels.

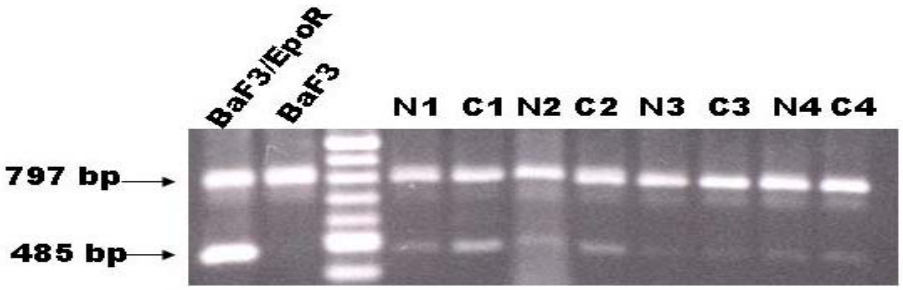


Figure 15. Normal human prostate tissue and primary human prostate cancer tissue express the EpoR gene. RNA was prepared from the BaF3/EpoR (positive control), BaF3 (negative control), and from four paired samples of normal (N) and cancerous (C) prostate epithelial tissue. RT-PCR was used to co-amplify a 485 bp fragment of the EpoR gene and a 797 bp fragment of the GAPDH gene from each of the prostate samples.

Some of these results have been published [22].

Additionally, the worked supported by this DOD grant stimulated other, related advances in our laboratory [23, 24].

KEY RESEARCH ACCOMPLISHMENTS

- Discovered reason for leaky Tet-On vector system. *Hyg* serves as a constitutive promoter.
- Determined that RT-PCR could not distinguish between sense and antisense transcript.
- Identified EpoR expression in all tumor samples examined. No relationship could be shown between expression levels, which were closely similar, and Gleason grade.
- Proved that EpoR on prostate cancer cells is functional and triggers signal transduction by the JAK/STAT pathway

REPORTABLE OUTCOMES

Papers published:

1. Chen C, Sytkowski AJ. Erythropoietin regulation of Raf-1 and Mek: evidence for a Ras-independent mechanism. Blood 2004; 104: 73-80.

2. Feldman L, Wang Y, Rhim JS, Bhattacharya N, Loda M, Sytkowski AJ. Erythropoietin Stimulates Growth and STAT5 Phosphorylation in Human Prostate Epithelial and Prostate Cancer Cells. Prostate. 2006. 66: 135-145. *Acknowledgement of support from DAMD17-03-1-0233 was*

inadvertently not included in the final paper. Nevertheless, DAMD17-03-1-0233 was a UmajorU source of funding for this work.

3. Debeljak N, Feldman L, Davis KL, Komel R, Sytkowski AJ. Variability in the Immunodetection of His-tagged Recombinant Proteins. Analytical Biochemistry 2006. 359: 216-223.

Manuscript submitted:

C. Gao and A. J. Sytkowski. Analysis of Constitutive Expression of the Erythropoietin Receptor Using a Tetracycline Inducible Expression Vector.

Book Published:

A. J. Sytkowski AJ. Erythropoietin: Blood Brain and Beyond. Wiley-VCH, Weinheim, 2004.

Abstract presentation:

L. Feldman, Y. Wang, J.S. Rhim, J.R. Busingye, A.J. Sytkowski. Human Prostate Epithelial and Prostate Cancer Cells Express both Erythropoietin and Functional Erythropoietin Receptors and Respond to Erythropoietin in Vitro. Presented at the annual meeting of the American Society of Hematology, Philadelphia, PA, December 6-10, 2002.

Cell lines:

LNCaP and PC-3 cell lines transfected with stable vectors expressing antisense human EpoR cDNA.

CONCLUSIONS

The erythropoietin receptor EpoR is expressed by human prostate cancer cells. These EpoR are functional and trigger a mitogenic response when activated by Epo. This has important clinical implications for prostate cancer patients receiving erythropoietin.

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APPENDIX - None

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